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Apigenin as an anti-quinolone-resistance antibiotic

Yuh Morimoto[a], Tadashi Baba[a], Takashi Sasaki[b], Keiichi Hiramatsu[a,*]

[a] Center of Excellence for Infection Control Science, Graduate School of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[b] Department of Bacteriology, Faculty of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

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ABSTRACT

We previously reported the first 'reverse antibiotic' (RA), nybomycin (NYB), which showed a unique antimicrobial activity against *Staphylococcus aureus* strains. NYB specifically suppressed the growth of quinolone-resistant *S. aureus* strains but was not effective against quinolone-susceptible strains. Although NYB was first reported in 1955, little was known about its unique antimicrobial activity because it was before the synthesis of the first quinolone ('old quinolone'), nalidixic acid, in 1962. Following our re-discovery of NYB, we looked for other RAs among natural substances that act on quinolone-resistant bacteria. Commercially available flavones were screened against *S. aureus*, including quinolone-resistant strains, and their minimum inhibitory concentrations (MICs) were compared using the microbroth dilution method. Some of the flavones screened showed stronger antimicrobial activity against quinolone-resistant strains than against quinolone-susceptible ones. Amongst them, apigenin (API) was the most potent in its RA activity. DNA cleavage assay showed that API inhibited DNA gyrase harbouring the quinolone resistance mutation *gyrA*(Ser84Leu) but did not inhibit 'wild-type' DNA gyrase that is sensitive to levofloxacin. An API-susceptible *S. aureus* strain Mu50 was also selected using agar plates containing 20 mg/L API. Whole-genome sequencing of selected mutant strains was performed and frequent back-mutations (reverse mutations) were found among API-resistant strains derived from the API-susceptible *S. aureus* strains. Here we report that API represents another molecular class of natural antibiotic having RA activity against quinolone-resistant bacteria.

1. Introduction

The emergence of bacteria resistant to antibiotics has become a worldwide concern. Although finding effective treatments for resistant strains is a top priority, few tools are available due to difficulty in the development of new antibiotics by the pharmaceutical industry. The history of antibacterial agents tells us that introduction of a new antibiotic is always followed by the appearance of resistant micro-organisms, with no exceptions [1]. Therefore, development of new drugs based on a completely novel concept is awaited.

We have previously reported nybomycin (NYB) as a candidate for such novel therapeutics [2]. NYB, produced by an actinobacterium, was first reported in 1955 [3]. At that time, quinolone-resistant bacteria had not been detected since clinical introduction of the quinolones did not start until the early 1960s [4]. Therefore, features of NYB with regard to quinolone resistance have not been studied. Our re-discovery of NYB as a 'reverse antibiotic' (RA) was brought about by screening of Actinobacteria cultures using quinolone-resistant meticillin-resistant *Staphylococcus aureus* (MRSA) as well as quinolone-susceptible *S. aureus* clinical strains. We looked for Actinobacteria supernatants that suppressed the growth of MRSA. The screening hit an actinobacterial strain, *Streptomyces hyalinum* MB891-A1, which produced a substance that had strong growth inhibition against MRSA strain Mu50. However, surprisingly the substance exhibited no activity against a meticillin-susceptible *S. aureus* (MSSA) type strain FDA 209P. The finding of this substance, identified as NYB, prompted us to determine the mode of antimicrobial activity of NYB. We noticed that MRSA strain Mu50 was resistant to quinolones but was susceptible to NYB, whereas the MSSA strain FDA 209P was quinolone-susceptible but NYB-resistant. NYB was found to specifically suppress the growth of quinolone-resistant *S. aureus* carrying mutation(s) in the quinolone resistance-determining region (QRDR) of *gyrA*, encoding the A subunit of DNA gyrase. However, NYB was not active against strains carrying intact or wild-type *gyrA* with no QRDR mutations. In addition, *S. aureus* mutant strains that acquired NYB resistance became quinolone-susceptible, and they were found to have reverse mutations to regain the 'wild-type' sequence in the QRDR. Biochemical analysis also revealed that NYB targets and inhibits the function of the mutated GyrA subunit.

* Corresponding author. Tel.: +81 3 5802 1040; fax: +81 3 5684 7830.
E-mail address: khiram06@juntendo.ac.jp (K. Hiramatsu).

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Thus, we found that the antimicrobial activity of NYB is complementary to that of quinolones. We therefore designated such a class of antibiotics represented by NYB as 'reverse antibiotics' (RAs).

During our attempt to screen for new RA candidates, we noticed an old report demonstrating a weak inhibitory effect of quercetin and related flavones on *Escherichia coli* DNA gyrase [5]. Flavones produced by plants are commonly used in folk remedies, thus their antibacterial activity has been studied previously [6]. However, the results were not impressive against the tested bacterial strains, including *S. aureus* [7,8]. Using quinolone-susceptible and -resistant *S. aureus* strains, we found that some flavones do have activity against *S. aureus*, and a few of them inhibited the growth of quinolone-resistant strains much better than that of susceptible ones.

Among such flavones, apigenin (API) was found to be the most conspicuous in its activity. In this report, we describe the mode of action of API and its property as another class of RA.

2. Materials and methods

2.1. Bacterial strains

A total of 36 *S. aureus* strains used in this study were isolated from Japan, the UK and the USA (Table 1). All of the strains have been described previously. Clinical *S. aureus* strains MS5935, MR5867, MS5952 and MR6009 and their stepwise quinolone-resistant mutants have been described previously [9]. *Staphylococcus aureus* NCTC 8325 and FDA 209P are MSSA type strains [2], and *S. aureus* N315 is a quinolone-susceptible MRSA [10]. KSA36, KBSA72 and KBSA56 are clinical MRSA isolated from Japanese hospitals in 2005 [2]. The remaining strains are clinical isolates with either reduced susceptibility [vancomycin-intermediate *S. aureus* (VISA)] ($n = 7$) or resistance [vancomycin-resistant *S. aureus* (VRSA)] ($n = 4$) to vancomycin.

2.2. Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the microbroth dilution method with an inoculum size of 10^4 CFU/well in a final volume of 100 μL. The procedure was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines [11].

API was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was used to prepare a 1024 mg/L API solution. Water containing up to 0.1 M NaOH was used for the preparation of quinolones. All solutions were diluted with cation-adjusted Mueller–Hinton broth (CA-MHB) [BD BBL™ Mueller–Hinton II Broth (Cation-Adjusted); BD Diagnostic Systems, Sparks, MD] for MIC measurement.

2.3. Mutant selection

Staphylococcus aureus strain Mu50 was inoculated in eight tubes containing 4 mL of tryptic soy broth (TSB) (BD Diagnostic Systems). After overnight incubation at 37 °C, the eight full-growth tubes were concentrated by 10-fold and were plated individually on a 150 mm × 15 mm Petri dish (BD Falcon™ 35-1058; BD Biosciences, Franklin Lakes, NJ) containing 75 mL of Mueller–Hinton agar medium (BD Diagnostic Systems) with 20 mg/L API. One colony from each plate was picked and subjected to further investigation.

2.4. PCR amplification and sequencing of the quinolone resistance-determining regions of gyrA and parC

Five sets of forward and reverse primers, respectively, were used: 5′-TTAGGTGATCGCTTTGGAAGATATAG-3′ and 5′-TACCATTGGTTCGAGTGTCG-3′ for *parC*; 5′-GGATTAAATGAACAAGGTATGACACCG-3′ and 5′-TAGTCATACGCGCTTCAGTATAACG-3′ for *gyrA*; 5′-CAGCGTTAGATGTAGCAAGT-3′ and 5′-CAGGACCTTCAATATCCTCC-3′ for *gyrB*; 5′-CGTAAGGACGTCTTGCCTGA-3′ and 5′-GGCTCATGACCAGCTGAACT-3′ for *rsbU*; and 5′-CCAGCAATTGGTAAAATCCAA-3′ and 5′-TGGTTCAAAACCAAGGGATG-3′ for *murC*. Nucleotide sequences were converted to amino acid sequences and were aligned with the amino acid sequences of the type II topoisomerases (ParC, GyrA and GyrB) of the parent strain Mu50 and reference strain FDA 209P to identify non-synonymous (or missense) mutations. Sequencing of the amplified DNA was done using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, Carlsbad, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Life Technologies).

2.5. Whole-genome sequencing and mutation detection

The whole genome sequences of in vitro-selected API-resistant mutants were determined using a MiSeq Genome Analyzer (Illumina, Inc., San Diego, CA). DNA extraction was performed using a QIAGEN Genome-tip System (QIAGEN, Hilden, Germany), and library preparations were performed using Nextera XT DNA sample preparation kits (Illumina, Inc.). Pools of eight samples were sequenced on the Illumina MiSeq platform, and 250-bp end reads were generated. Reads from bacterial strains were then mapped to the reference whole genome sequence of strain Mu50 (GenBank accession no. NC_002758.2) and mutations were identified using Tablet v.1.13.04.22 (The James Hutton Institute, Aberdeen, UK). The identified single nucleotide polymorphism were then verified by re-sequencing using an Applied Biosystems 3730 Capillary Sequencer (Applied Biosystems Ltd., Tokyo, Japan) for each locus.

2.6. Cytokilling assay

Overnight cultures of tested strains in TSB were diluted and ca. 0.5 mL of 10^6 CFU/mL was transferred into 4.5 mL of fresh CA-MHB containing 0, 8, 16, 20, 24 or 32 mg/L API. Test tubes were incubated with gentle shaking and 0.1 mL portions of the culture were harvested to a drug-free plate at 0, 0.5, 1, 3, 5, 7 and 24 h. The grown colonies were counted and plotted on a graph. The cultivation temperature of the test strains was 37 °C for Mu50 and KBSA56. These strains were also tested at a cultivation temperature at 43 °C.

2.7. Demonstration of gyrase inhibition by DNA cleavage assay

The assay was based on an established system as described by Fisher and Pan [12]. Briefly, supercoiled plasmid DNA pTWV228 (a pBR322 derivative) (TaKaRa Bio Inc., Kusatsu, Japan) was pre-incubated in the presence of either API or levofloxacin, followed by addition of GyrB in combination with either the wild-type or mutated GyrA proteins and then incubated at 25 °C for 1 h. The following analyses, including determination of IC_{50} values (50% inhibitory concentration), were carried out as described previously [2].

3. Results

By testing 104 flavones, we found at least three flavones that shared preferential antimicrobial activity against MRSA Mu50 compared with MSSA FDA 209P, including API, myricetin-3,7,3′,4′-tetramethyl ether and isopratol. Their MICs are shown in Table 2. Among the three flavones, API showed the most typical MIC profile of a RA. We therefore decided to study API.

Table 1
Staphylococcus aureus strains used in this study.

Strain[a]	Year of isolation	Country (state) of isolation	Category	Reference
FDA 209P (NCTC 7447, ATCC 6538)	1948[b]	UK	MSSA	[2]
Mu50 (ATCC 700699, NRS1)	1996	Japan	MRSA (VISA)	[10]
MS5935	1996[b]	Japan	MSSA	[9]
MS5935 1st step	1996[b]	Japan	MSSA	[9]
MS5935 2nd step	1996[b]	Japan	MSSA	[9]
MS5935 3rd step	1996[b]	Japan	MSSA	[9]
MS5935 4th step	1996[b]	Japan	MSSA	[9]
MR5867	1996[b]	Japan	MRSA	[9]
MR5867 1st step	1996[b]	Japan	MRSA	[9]
MR5867 2nd step	1996[b]	Japan	MRSA	[9]
MR5867 3rd step	1996[b]	Japan	MRSA	[9]
MR5867 4th step	1996[b]	Japan	MRSA	[9]
MS5952	1996[b]	Japan	MSSA	[9]
MS5952 1st step	1996[b]	Japan	MSSA	[9]
MS5952 2nd step	1996[b]	Japan	MSSA	[9]
MS5952 3rd step	1996[b]	Japan	MSSA	[9]
MR6009	1996[b]	Japan	MRSA	[9]
MR6009 1st step	1996[b]	Japan	MRSA	[9]
MR6009 2nd step	1996[b]	Japan	MRSA	[9]
MR6009 3srd step	1996[b]	Japan	MRSA	[9]
MR6009 4th step	1996[b]	Japan	MRSA	[9]
NCTC 8325	1943	UK	MSSA	[2]
N315	1982	Japan	MRSA	[10]
VRS1	2002	USA	VRSA	[2]
HIP09143	2000	USA	MRSA (VISA)	[2]
HIP09662	2000	USA	MRSA (VISA)	[2]
NRS118	2002	USA	MRSA (VISA)	[2]
VRS4	2005	USA	VRSA	[2]
HIP07920	1998	USA	MRSA (VISA)	[2]
HIP5836 (NJ)	1997	USA	MRSA (VISA)	[2]
KSA36	2005	Japan	MRSA	[2]
VRS3a	2004	USA	VRSA	[2]
KBSA72	2005	Japan	MRSA	[2]
VRS5	2005	USA	VRSA	[2]
KBSA56	2005	Japan	MRSA	[2]
HIP07930	1999	USA	MRSA (VISA)	[2]

MSSA, meticillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; VISA, vancomycin-intermediate *S. aureus*; VRSA, vancomycin-resistant *S. aureus*.
[a] Alternative names for each strain are given in parenthesis if they have been used quite frequently in publications.
[b] Year of accession (not year of isolation).

Table 2
Minimum inhibitory concentrations (MICs) of three flavones against quinolone-resistant *Staphylococcus aureus* strain Mu50 and quinolone-susceptible *S. aureus* FDA 209P.

Compound	MIC (mg/L)	
	Mu50	FDA 209P
Apigenin	4	>128
Myricetin-3,7,3′,4′-tetramethyl ether	8	128
Isopratol	16	>128
Norfloxacin	128	0.25
Levofloxacin	8	≤0.06

3.1. Anti-quinolone-resistance activity of apigenin

The antibacterial activity of API was determined for quinolone-susceptible and -resistant *S. aureus* strains and was compared with that of several fluoroquinolones (norfloxacin, ofloxacin, levofloxacin, ciprofloxacin, sparfloxacin and tosufloxacin) and NYB (Table 3). Although the growth inhibitory activity was 16-fold weaker than NYB, API showed the feature of a RA, i.e. stronger antimicrobial activity against quinolone-resistant *S. aureus* Mu50 than against quinolone-susceptible FDA 209P. To determine the relationship between API susceptibility and quinolone resistance, isogenic sets of quinolone-resistant mutant strains were tested. The four parent strains (MS5935, MS5952, MR5867 and MR6009) are clinical isolates, and their stepwise quinolone-resistant mutants are their in vitro derivatives [9]. Table 3 shows that all four parent strains having wild-type ParC and GyrA proteins were susceptible to the six quinolone antibiotics but were resistant to API and NYB. The activity of quinolones decreased with the accumulation of mutations in the QRDRs of ParC and GyrA. API gained activity with the procession of quinolone resistance. The MIC of API dropped from 128 mg/L to ≤8 mg/L with *gyrA*(Ser84Leu) mutation. The third-step mutant of MR6009 with *parC*(Ser80Tyr, Glu84Lys) double mutations also showed increased susceptibility towards API.

Subsequently, a total of 17 clinically isolated strains that were ether MSSA, MRSA, VISA or VRSA with variable types of QRDR mutations were tested (Table 4). Three strains having wild-type QRDRs showed resistance to API. The other 14 strains having *gyrA*(Ser84Leu) mutation showed an API MIC of ≤4 mg/L. Three strains that acquired *gyrA*(Ser84Leu, Glu88Gly) mutations showed an MIC of 0.5 mg/L and were the most susceptible to API. The reverse pattern of MIC values between quinolones and NYB was once again observed with API (Fig. 1).

3.2. Cytokilling assay

Fig. 2a illustrates the time-dependent cytokilling activity of API against 17 *S. aureus* strains that were used for the MIC measurement described above. In the experiment, 20 mg/L API was used. The results roughly gave three patterns of killing curves: pattern 1 represents API to have no effect on bacterial growth (NCTC 8325, N315); pattern 2 represents API to have some effect to inhibit bacterial growth but strains increased the number of cells after 7 h of incubation (FDA 209P, HIP09662, NRS118, HIP5836, NRS118,

Table 3
Apigenin (API) is active against *Staphylococcus aureus* strains with a *gyrA*(Ser84Leu) mutation.

Strain[a]	MIC (mg/L)[b]								QRDR AA substitutions[c]	
	NOR	OFX	LVX	CIP	TSX	SPX	NYB	API	ParC	GyrA
FDA 209P (NCTC 7447, ATCC 6538)	**0.25**	**0.125**	**≤0.06**	**0.125**	**≤0.06**	**≤0.06**	64	128	–	–
Mu50 (ATCC 700699, NRS1)	128	16	8	32	>128	16	**0.25**	**4**	Ser80Phe	Ser84Leu
MS5935	**1**	**0.25**	**0.125**	**0.25**	**0.125**	**≤0.06**	>64	128	–	–
MS5935 1st step	16	**1**	**0.5**	**2**	**1**	**≤0.06**	>64	128	Ser80Phe	–
MS5935 2nd step	128	16	8	32	64	16	**1**	**4**	Ser80Phe	Ser84Leu
MS5935 3rd step	128	128	64	128	>128	32	**1**	**4**	Ser80Phe, Glu84Lys	Ser84Leu
MS5935 4th step	128	>128	>128	128	>128	128	32	**4**	Ser80Phe, Glu84Lys	Ser84Leu, Glu88Val
MR5867	**1**	**0.25**	**0.125**	**0.25**	**≤0.06**	**≤0.06**	>64	>128	–	–
MR5867 1st step	16	**1**	**0.5**	**2**	**0.25**	**≤0.06**	>64	128	Glu84Lys	–
MR5867 2nd step	64	16	8	32	8	8	**0.25**	8	Glu84Lys	Ser84Leu
MR5867 3rd step	>128	64	16	128	>128	16	**0.25**	**4**	Ser80Phe, Glu84Lys	Ser84Leu
MR5867 4th step	>128	>128	>128	128	>128	128	**0.25**	**4**	Glu84Lys, Ser80Phe	Ser84Leu, Glu88Lys
MS5952	**1**	**0.25**	**0.125**	**0.5**	**0.5**	**<0.06**	>64	128	–	–
MS5952 1st step	16	**1**	**0.5**	**2**	**2**	**0.125**	>64	128	Ser80Tyr	–
MS5952 2nd step	16	8	**4**	8	8	8	**0.5**	8	Ser80Tyr	Ser84Leu
MS5952 3rd step	128	64	16	64	>128	16	**0.5**	**2**	Ser80Tyr, Ala116Val	Ser84Leu
MR6009	**1**	**0.25**	**0.125**	**0.25**	**<0.06**	**<0.06**	>64	128	–	–
MR6009 1st step	16	**1**	**0.5**	**2**	**1**	**<0.06**	>64	128	Ser80Tyr	–
MR6009 2nd step	64	16	**4**	32	16	**4**	>64	128	Ser80Tyr	Glu88Lys
MR6009 3rd step	>128	32	16	128	>128	8	**4**	**4**	Ser80Tyr, Glu84Lys	Glu88Lys
MR6009 4th step	>128	>128	>128	128	>128	128	**0.25**	**4**	Ser80Tyr, Glu84Lys	Ser84Leu Glu88Lys

MIC, minimum inhibitory concentration; NOR, norfloxacin; OFX, ofloxacin; LVX, levofloxacin; CIP, ciprofloxacin; TSX, tosufloxacin; SPX, sparfloxacin; NYB, nybomycin; QRDR, quinolone resistance-determining region; AA, amino acid.

[a] Four isogenic sets of strains were used.

[b] MICs ≤ 4 mg/L are in bold.

[c] '–' indicates no AA substitution.

Table 4
Minimum inhibitory concentrations (MICs) of six quinolones, nybomycin (NYB) and apigenin (API) against 17 clinically isolated *Staphylococcus aureus* strains.

Strain	Country	Year of isolation		AA substitution[a]		MIC (mg/L)[b]							
				ParC	GyrA	NOR	OFX	LVX	CIP	TSX	SPX	NYB	API
NCTC 8325	UK	1943	MSSA	w/t	w/t	**1**	**0.25**	**0.125**	**0.25**	**≤0.06**	**0.125**	>64	>128
FDA 209P	UK	1948	MSSA	w/t	w/t	**0.25**	**0.125**	**≤0.06**	**0.125**	**≤0.06**	**≤0.06**	64	128
N315	Japan	1982	MRSA	w/t	w/t	**1**	**0.25**	**0.125**	**0.25**	**≤0.06**	**≤0.06**	64	>128
Mu50	Japan	1996	MRSA	Ser80Phe	Ser84Leu	128	16	8	32	>128	16	**0.25**	**4**
VRS1	USA	2002	VRSA	Ser80Phe	Ser84Leu	>128	32	16	128	**4**	8	**0.125**	**4**
HIP07920	USA	1998	MRSA	Glu84Lys	Ser84Leu	16	16	8	16	**4**	16	**0.25**	**4**
HIP5836 (NJ)	USA	1997	MRSA	Ser80Tyr	Ser84Leu	128	64	8	16	32	32	**0.5**	**2**
HIP07930	USA	1999	MRSA	Ile45Met, Ser80Phe	Ser84Leu	16	16	8	16	16	16	**0.25**	**4**
NRS118	USA	2002	MRSA	Ser80Phe, Ser81Pro	Ser84Leu	128	64	32	32	>128	32	**0.25**	**4**
VRS3a	USA	2004	VRSA	Ser80Tyr, Glu84Lys	Ser84Leu	128	32	16	64	>128	16	**0.125**	**2**
HIP09662	USA	2000	MRSA	Ser80Phe, Glu84Gly	Ser84Leu	64	32	32	32	>128	16	**1**	**4**
VRS5	USA	2005	VRSA	Ser80Tyr, Glu84Gly	Ser84Leu	>128	64	32	>128	>128	16	**≤0.06**	**2**
HIP09143	USA	2000	MRSA	Ser80Phe, Glu84Lys	Ser84Leu	>128	64	32	64	>128	16	**0.25**	**2**
VRS4	USA	2005	VRSA	Ser80Tyr, Glu84Gly	Ser84Leu, Glu88Lys	>128	>128	>128	>128	>128	>128	**≤0.06**	**2**
KSA36	Japan	2005	MRSA	Ser80Tyr	Ser84Leu, Glu88Gly	>128	>128	>128	128	>128	128	**≤0.06**	**0.5**
KBSA72	Japan	2005	MRSA	Ser80Tyr, Glu84Lys	Ser84Leu, Glu88Gly	>128	>128	>128	128	>128	128	**≤0.06**	**0.5**
KBSA56	Japan	2005	MRSA	Ser80Tyr, Glu84Val	Ser84Leu, Glu88Gly	128	>128	>128	64	>128	>128	**≤0.06**	**0.5**

AA, amino acid; NOR, norfloxacin; OFX, ofloxacin; LVX, levofloxacin; CIP, ciprofloxacin; TSX, tosufloxacin; SPX, sparfloxacin; NYB, nybomycin; API, apigenin; MSSA, meticillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; VRSA, vancomycin-resistant *S. aureus*.

[a] 'w/t' stands for wild-type (identical to strain FDA 209P).

[b] MICs ≤ 4 mg/L are in bold.

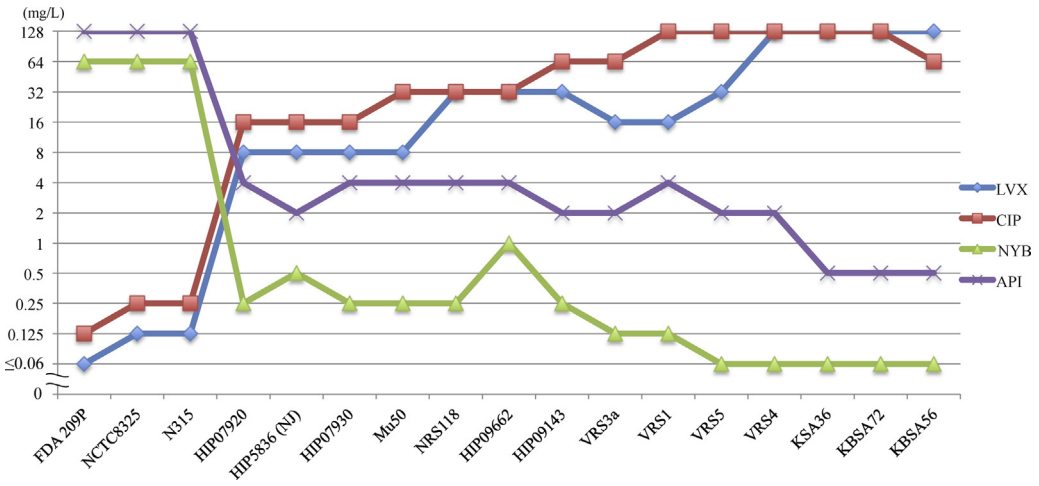

Fig. 1. Proportions of susceptibility to quinolones (LVX and CIP) and 'reverse antibiotics' (RAs) (NYB and API) against 17 *Staphylococcus aureus* clinical strains with various genotypes of type II topoisomerase. LVX, levofloxacin; CIP, ciprofloxacin; NYB, nybomycin; API, apigenin. The reverse pattern of minimum inhibitory concentrations between quinolones and NYB was also observed with API.

Mu50, VRS4, VRS5, HIP07930); whereas the curves of the pattern 3 strains (HIP09143, VRS1, VRS3a, HIP07920, KBSA72, KBSA56, KSA36) decreased with time. Bacterial CFU of KBSA72, KBSA56 and KSA36 decreased >10^3-fold after 24 h.

For a more precise investigation, Mu50 and KBSA56 were inoculated into CA-MHB containing 0, 8, 16, 20, 24, 28 and 32 mg/L API and CFU were counted after 0, 0.5, 1, 3, 5, 7 and 24 h of incubation. At an incubation temperature of 37 °C, the numbers of Mu50 cells remained in the same order of the starting time after 7 h of incubation for all API concentrations. The cell number remained the same 10^5 order after 24 h in the presence of 20 mg/L, and <2.0×10^6 CFU in the presence of 24 mg/L and 28 mg/L API. In the presence of 8, 16 and 32 mg/L API, however, the viable cell count increased from 1.73×10^5 to 7.20×10^7, from 1.81×10^5 to 6.50×10^7, and from 2.31×10^5 to 5.13×10^6, respectively, after 24 h. Although the differences are small, 20 mg/L was the most effective concentration to inhibit the cell growth of Mu50. API reduced the number of bacterial cells of KBSA56 having four mutations in topoisomerases: Ser80Tyr and Glu84Val in ParC; and Ser84Leu and Glu88Gly in GyrA. The viable cell number decreased from 2.36×10^5 to 1.15×10^4 at 7 h, and to 1.30×10^2 at 24 h (32 mg/L API). Unlike against Mu50, API appeared to be bactericidal to KBSA56, decreasing the number of cells to <10^3 with a concentration of ≥20 mg/L. Furthermore, the cytokilling activity of API against Mu50 and KBSA56 was tested at an incubation temperature of 43 °C. API also inhibited bacterial growth of Mu50 at 43 °C. The number of bacteria remained in the same order until 7 h, except at an API concentration of 8 mg/L, which allowed a 10-fold increase in CFU by 7 h of incubation. After 24 h of incubation, all Mu50 cultures with API exceeded 10^6 CFU/mL. The drug-free culture after 24 h of incubation was 3.5×10^8 CFU/mL. API gained antimicrobial activity at the lower concentrations against KBSA56. Viable cell counts after exposure to 8 mg/L and 16 mg/L API were >10^4 times lower than that after 24 h of drug-free incubation at 37 °C. These results indicated that API is either bacteriostatic or bactericidal depending on the target *S. aureus* strains. The cytokilling activity of API was more pronounced against KBSA56 at the raised incubation temperature of 43 °C (Fig. 2b). The doubly mutated GyrA (and ParC) may have a temperature-sensitive phenotype and may become more sensitive to API at a raised temperature.

3.3. The target of action of apigenin

To demonstrate that API acts on GyrA with Ser84Leu mutation, a topoisomerase DNA cleavage assay system was employed using wild-type as well as mutated DNA gyrase subunit A [12]. As expected, API specifically accumulated cleaved form of the substrate DNA when the mutated form of GyrA was used (Fig. 3), indicating direct inhibition of the mutated DNA gyrase in the presence of API. Note that levofloxacin inhibited the wild-type gyrase but not the mutant, showing that the quinolone and API have mutually reverse effects on gyrase, which would explain the reverse correlation of MICs of quinolones and API (Table 4). API inhibited mutated gyrase with an IC_{50} value of 29.6 μM, whereas the value for NYB was 6.7 μM [1]. The weaker inhibition of mutated gyrase by API than NYB reflects the difference in the MICs between the two RA agents.

3.4. Apigenin mutant selection and reverse mutants

To investigate weather API helps generate 'reverse mutation' allowing the mutated QRDR to return to 'wild-type', mutants were selected from API-susceptible Mu50 strain using an API-containing agar plate.

Eight independent Mu50 cultures were plated on API-containing solid medium in Falcon 150 mm × 15 mm dishes. According to the results of the cytokilling assay, the API concentration was adjusted to 20 mg/L. As expected, *gyrA* reverse mutant strains were detected in two of eight dishes, indicating that API also has the feature of a RA. However, we found that API selection causes some other mutations: whole-genome sequencing revealed that one strain acquired mutation in *gyrA* outside of the QRDR (Phe480Val) and four strains acquired mutation in *gyrB* (Leu457Phe, Glu477Asp and Ile539Phe). One strain acquired mutation in *murC* (Arg375Ile). One of the four strains with *gyrB*(Leu457Phe) mutation was observed to have two peaks on the 476th nucleotide of *rsbU* (C and T). The strain had a greater number of C (the original nucleotide in Mu50), than T. A mutation of the 476th nucleotide from C to T causes amino acid substitution of Pro159Leu.

MICs of the eight API-resistant strains were measured. The back-mutated strains had increased MICs for API and NYB and decreased MICs for the six tested quinolones (Table 5). Six strains without back-mutation had an increased API MIC, but they remained resistant to quinolones and susceptible to NYB (MICs remained <2 mg/L).

These analyses indicated that API has a RA feature of allowing back-mutation in the *gyrA* QRDR. However, unlike NYB, API appears to permit a few other genetic changes that confer resistance to its rather mild antibacterial activity.

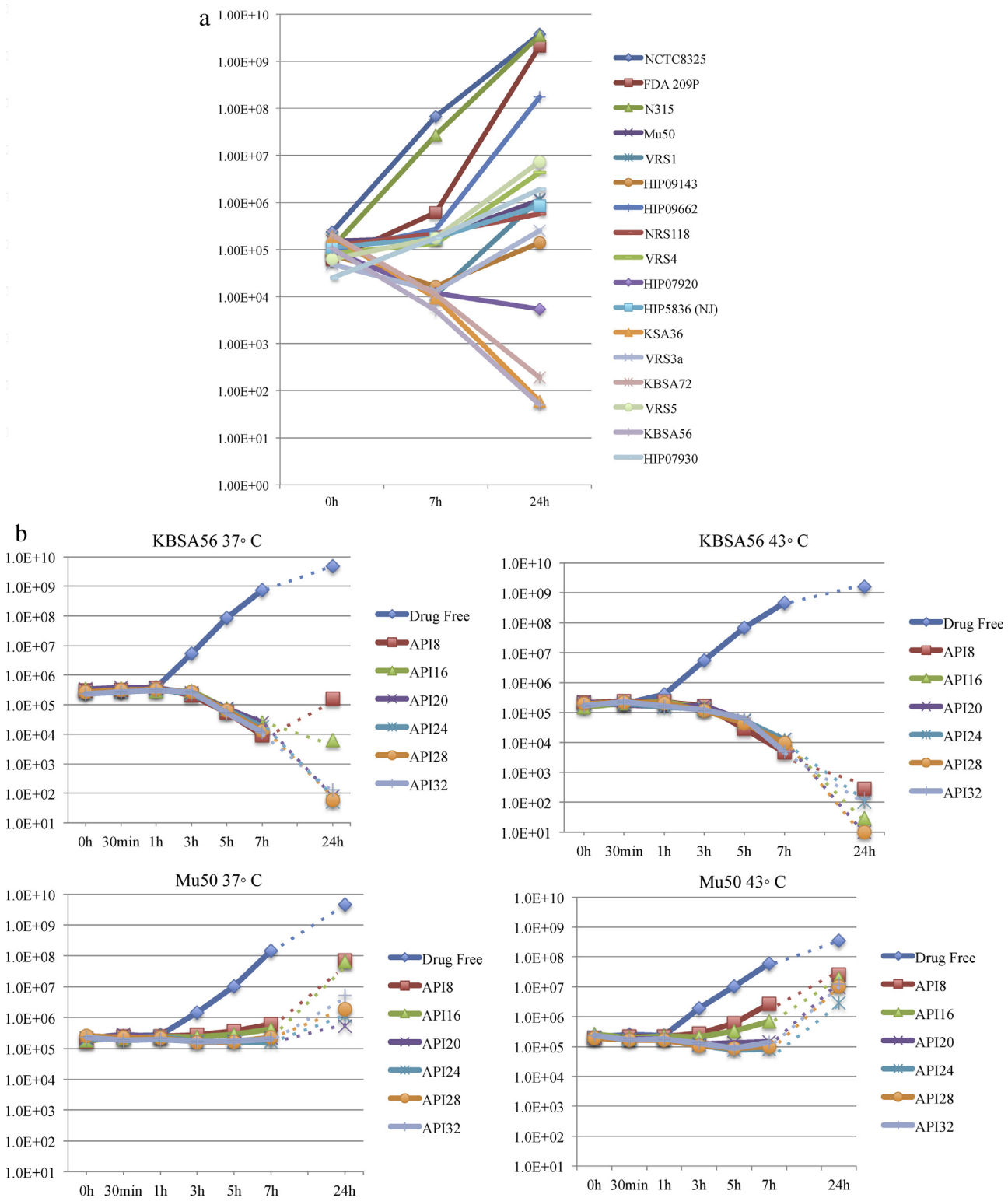

Fig. 2. (a) Cytokilling assay of 20 mg/L apigenin (API) against 17 clinically isolated *Staphylococcus aureus* strains. The results roughly gave three patterns of killing curves: pattern 1 represents API to have no effect on bacterial growth (NCTC 8325, N315); pattern 2 represents API to have some effect to inhibit bacterial growth but strains increased the number of cells after 7 h of incubation (FDA 209P, HIP09662, NRS118, HIP5836 (NJ), NRS118, Mu50, VRS4, VRS5, HIP07930); whereas the curves of the pattern 3 strains (HIP09143, VRS1, VRS3a, HIP07920, KBSA72, KBSA56, KSA36) decreased with time. Bacterial CFU of KBSA72, KBSA56 and KSA36 decreased >10^3-fold after 24 h. (b) Cytokilling curves of graded antibiotic concentration against Mu50 and KBSA56. Mu50 and KBSA56 were inoculated into cation-adjusted Mueller–Hinton broth containing 0, 8, 16, 20, 24, 28 or 32 mg/L API and CFU were counted after 0, 0.5, 1, 3, 5, 7 and 24 h of incubation. The numbers of Mu50 cells remained in the same order of the starting time after 7 h of incubation for all API concentrations at an incubation temperature of 37 °C. API reduced the number of the bacterial cells of KBSA56 having Ser84Leu and Glu88Gly mutations in *gyrA*.

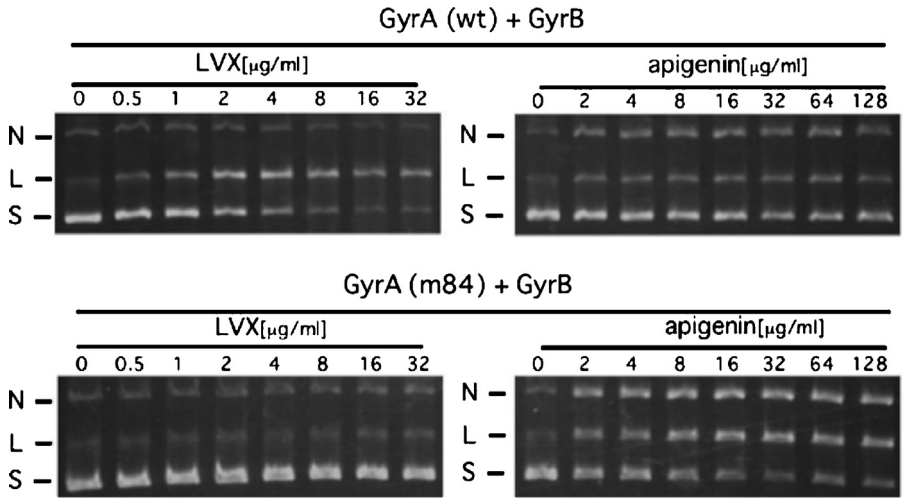

Fig. 3. In vitro DNA cleavage assay shows that apigenin specifically inhibits the mutated form of DNA gyrase, whereas the mutant is resistant to levofloxacin (LVX), one of the most frequently used quinolones. In the figure, 'S' represents supercoiled substrate plasmid DNA pTWV228, 'L' is the linearised form of the plasmid, whereas 'N' indicates the nicked form of the plasmid. The decrease of intensity in population 'S' and corresponding accumulation of 'L' and 'N' forms means failure of the DNA religation activity by effective drugs after cleavage of the substrate DNA by DNA gyrase. wt, wild-type.

Table 5
Amino acid substitutions and minimum inhibitory concentrations (MICs) of six quinolones, nybomycin (NYB) and apigenin (API) against *Staphylococcus aureus* Mu50 mutants selected by 20 mg/L API.

Strain	AA substitution[a]			MIC (mg/L)[b]							
	gyrA	*gyrB*	Other gene	NOR	OFX	LVX	CIP	TSX	SPX	NYB	API
Parent	–	–	–	128	16	8	32	>128	16	**0.25**	**4**
1	–	Leu457Phe	*rsbU*(Pro159Leu)	64	16	8	32	>128	16	**1**	>128
2	–	–	*murC* (Arg375Ile)	128	16	8	64	>128	16	**0.25**	128
3	Phe480Val	–	–	128	32	16	32	>128	32	**1**	>128
4	Leu84Ser	–	–	8	**2**	**1**	**2**	**0.5**	**0.25**	>64	>128
5	–	Ile539Phe	–	64	32	16	32	>128	16	**2**	>128
6	Leu84Ser	–	–	8	**2**	**1**	**2**	**0.5**	**0.25**	>64	>128
7	–	Glu477Asp	–	128	16	8	32	>128	64	**1**	>128
8	–	Ile539Phe	–	128	16	8	32	>128	16	**2**	>128

AA, amino acid; NOR, norfloxacin; OFX, ofloxacin; LVX, levofloxacin; CIP, ciprofloxacin; TSX, tosufloxacin; SPX, sparfloxacin.
[a] '–' indicates no AA substitution.
[b] MICs ≤ 4 mg/L are in bold.

4. Discussion

From ancient times, flavonoid-producing plants have been used in folk and traditional medicine [13,14]. As a member of the flavones, API is found in some plants, including parsley, olive leaves and, one of the most popular medicinal plants, chamomile [13,15,16]. Several groups have studied the activity of flavones, including API, against *S. aureus* [6]. In in vitro-based studies, several modes of action were reported [17–19]. However, the activity of API towards microbes was determined as marginal or none [20], except for one report presenting MICs of 3.9–15.6 mg/L against *S. aureus* strains [21].

In this study, screening was carried out based on the concept of RA against quinolone-resistant bacteria [2]. Therefore, not only quinolone-susceptible type strains but also quinolone-resistant strains as well as those resistant to multiple antibiotics were included in the *S. aureus* MIC panel. As a result, we have discovered that API has an MIC of 2–4 mg/L against *S. aureus* strains when they carry Ser84Leu mutation in *gyrA*. Furthermore, API exerted a bactericidal effect on three clinically isolated MRSA strains (KSA36, KBSA72 and KBSA56). The MIC of 0.5 mg/L recorded for the three strains was the lowest of API for any clinical *S. aureus* strains we have so far tested.

Cytokilling assay revealed that API inhibits proliferation of *S. aureus* strains carrying Ser84Leu mutation in *gyrA* (Table 4). API reduced the number of viable cells for several *S. aureus* strains (KSA36, KBSA72 and KBSA56). They were isolated in 2005 from hospitals in the eastern and western sides of Japan. They were characteristic in carrying *gyrA*(Glu88Gly) mutation. In general, Glu88Gly mutation in *gyrA* is not common. We have not found any other isolates in our collection nor in the BLAST search database of the National Center for Biotechnology Information (NCBI) website.

KBSA56 was used for further cytokilling investigation. The temperature was set first at 37 °C and then at 43 °C. API reduced CFU of KBSA56 both at 37 °C and 43 °C. The decrease in CFU was greater at 43 °C, especially at lower API concentrations (8 mg/L and 16 mg/L).

Regarding the cytokilling activity of flavones, there is a discussion that flavones 'glue' bacterial cells and thus reduce the number of CFU without killing the cells [6]. This might also be happening on the tested cells in our panel. However, aggregation alone was not enough to explain the patterns of the killing curve (Fig. 2b). CFU of the strains carrying amino acid substitution Glu88Gly decreased from 10^5 to <10 when exposed to API. Tighter binding of API to the doubly mutated GyrA may be the reason for cytokilling of the cell through prolonged inactivation of gyrase function.

API mutant selection was performed using Mu50. Two back-mutated strains were obtained in a total of eight samples. Unlike NYB, API appears to permit bacterial cells several different types of mutations to escape from its activity. Four API-resistant strains acquired mutation in *gyrB* and became resistant both to quinolones and API. Against these mutants, however, the NYB MIC remained <2 mg/L against all of the API-resistant Mu50-derived strains.

Flavonoids were produced by plants that covered the earth's surface producing thick forests and wetlands in the Carboniferous period (ca. 359–199 million years ago). Almost all bacteria in old times are considered to have been exposed to flavonoids and must have become resistant to them. Quinolones were first introduced in the 1960s by humans. Therefore, it is curious why API has antimicrobial activity against quinolone-resistant *S. aureus* strains, whereas it is ineffective against *S. aureus* susceptible to quinolones. Plants and microbes have shared the living environment since ancient times, thus some flavones might have been used to check the overgrowth of the cohabiting micro-organisms. The first land-adapted plants appeared during the mid-Palaeozoic era, ca. 480–360 million years ago [22]. It is curious to speculate that API was once much more active against the soil bacteria 400 million years ago, but accumulated QRDR mutations since then to have become resistant to flavones. Then, humans synthesised the quinolone antibiotics for use against flavone-resistant bacteria, which reduced the number of 'wild-type' strains and increased the number of 'quinolone-resistant mutant' strains having similar topoisomerases of the ancient bacteria. Thus, quinolones and flavones might have been lined historically as mutually complementary RAs.

This finding of API and NYB as natural groups of RAs against quinolone resistance suggests that the use of RAs may open a new era of chemotherapy that has long been suffering from the increase in drug-resistant bacteria. RAs in nature would not be confined to quinolone resistance. Further investigation would reveal more kinds of RAs in nature. Rational use of various RAs would allow us finally resolve the problem of the vicious cycle between antibiotics and antibiotic resistance.

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